Title: Exploiting Multisite Gateway and pENFRUIT plasmid collection for fruit genetic engineering.

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Abstract

The Multisite Gateway cloning techniques based on homologous recombination facilitate the combinatorial assembly of basic genetic pieces (i.e. promoters, CDS, terminators) into gene expression or gene silencing cassettes. pENFRUIT is a collection of multisite triple gateway Entry vectors dedicated to genetic engineering in fruits. It comprises a number of fruit-operating promoters as well as C-terminal tags adapted to the gateway standard. In this way, flanking regulatory/labeling sequences can be easily gateway-assembled with a given gene-of-interest for its ectopic expression or silencing in fruits. The resulting gene constructs can be analyzed in stable transgenic plants or in transient expression assays, the latter allowing fast testing of the increasing number of combinations arising from multisite methodology. A detailed description of the use of multisite cloning methodology for the assembly of pENFRUIT elements is presented.

1. Introduction

Fruits are natural factories of edible, health-promoting substances. Increasing the concentration and/or broadening the spectrum of added-value compounds produced in fruits is a challenging biotechnological goal. In fruit biotechnology a good experimental design includes testing various expression cassettes controlled by different promoters, which should be active at a given development phase or tissue **(*1*-*3*)**. The Gateway cloning system, based on homologous recombination **(*4*)**, has become a popular alternative to traditional cloning methods based on the T4 ligase for constructing expression cassettes, and thus several destination vector collections for plants are currently available to the scientific community **(*5*-*7*)**. Multisite Gateway system is a variation that enables to construct in a vector up to four fragments "*in tandem*" by using a single recombination reaction. In this way, a collection of vectors can be developed and combined with a particular Gene of Interest (GOI) **(*8, 9*)**. Entry vectors collections are designed to facilitate the combinatorial assembly of basic genetic pieces (i.e. promoters, CDS, terminators) into gene expression or gene silencing cassettes. pENFRUIT is a Entry vectors collection specifically dedicated to genetic engineering in fruits, and made available for the research community through a plasmid repository **(*10*)**. pENFRUIT collection comprises a number of fruit-operating promoters covering a wide range of tissues and developmental stages, as well as a number of C-terminal tags for the labeling of the protein-of-interest. All together, pENFRUIT provides a total of seventy-eight possible combinations for the expression of a gene of interest in the tomato fruit. Moreover, pENFRUIT vectors can be used for hpRNAi strategies with the sole requirement of a traditional cloning step using restriction enzymes.

Concomitantly with the development of increasingly versatile cloning methods, the need for simple and rapid analysis of gene function has also increased, so the use of transient expression assays has bloomed as an alternative to the analysis of stable transformants **(*11-13*)**. Transient expression allows the production of recombinant proteins in a short time and does not require the use of a marker gene for selecting transformed plants. This results smaller expression vectors with more efficient ligation reactions and bacterial transformation. Agroinjection is a transient methodology which directs transient expression of foreign genes directly in the fruit **(*11*)**. Injection of diluted *Agrobacterium* cultures at different immature stages resulted in transient expression of reporter genes in placenta, gel and inner pericarp layers.

In this chapter we describe in detail the methodology employed for the construction of new gene expression cassettes operating in the fruit using gateway-based pENFRUIT vector collection. Furthermore, the testing of selected constructs in fruits using transient expression (agroinjection) is also described.

2. Materials

2.1. *att*B4r/*att*B3r GOI PCR amplification and BP cloning

1. HPLC or PAGE purified attB4r and attB3r flanking oligonucleotides (Invitrogen, Carlsbad, CA, USA).Advantage HD Polymerase Mix (Clontech, Mountain View, CA, USA) .dNTP mixture (10 mM each dNTP) and sterilized water.

2. For gel extraction and purification: 1 % agarose TAE 1X (40 mM Tris-Acetate and 1 mM Na_2EDTA.) For PEG purification: 1X TE Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). 30% PEG 8000/30 mM $MgCl_2$ supplied with the BP Clonase™ II

Enzyme Mix kit (Invitrogen, Carlsbad, CA, USA) and a microcentrifuge (Micro Centaur, SANYO).

3. MultiSite Gateway® Pro pDONR221 P4r-P3r (Invitrogen, Carlsbad, CA, USA) vector for each *att*B4r/*att*B3r flanked PCR product. BP Clonase™ II Enzyme Mix kit (Invitrogen, Carlsbad, CA, USA). 1X Tris-EDTA (TE) Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Proteinase K solution (2 µg/µL) as included the BP clonase kit (Invitrogen, Carlsbad, CA, USA).

4. A Thermolyne Dri-Bath Model 17600 (Thermo Fisher Scientific, Hampton, USA) thermo-block or a water bath (Precisterm S-385) set at 37 ºC.

2.2. Cloning of the GOI in pEFS4r-3r and pEFS3-2 vectors

1. DNA containing your GOI sequence and pEFS4r-3r and pEFS3-2 vectors from Addgene plasmid repository (http://www.addgene.org/pgvec1). Two different restriction enzymes recognizing the polylinker site *SacI, SmaI or XhoI* and their buffers (New England Biolabs, Hitchin, UK).

2. T4 DNA Ligase (Invitrogen, Carlsbad, CA, USA) and a thermo block set at 24 ºC, 37 ºC and 65 ºC

2.3. *E. coli* cell transformation and culture

1. One Shot® TOP10 or One Shot® Mach1™ T1R chemically competent *E. coli* kit (Invitrogen, Carlsbad, CA, USA). Sterile LB liquid medium and solid LB agar (LBA) plates containing 50 µg/mL Kanamycin in case of Entry clones (BP reaction) or 50 µg/mL Spectinomycin for Expression clones (LR reaction).

2. E.Z.N.A.® Plasmid Mini Kit II (Omega Bio-tek, Doraville, GA, USA). A 42 °C water bath (Precisterm S-385) and 37 °C shaking incubators.

2.4. Triple Gateway LR recombination reaction

1. LR Clonase™ II Plus Enzyme Mix kit (Invitrogen, Carlsbad, CA, USA). Keep at -20°C or -80°C until immediately before use.

2. Miniprep-purified plasmid DNA of Entry clones pEF1-4, pEF4r-3r (or pEFS4r-3r) and pEF3-2 (or pEFS3-2) (supercoiled, 10 fmoles). Twenty fmoles of purified plasmid DNA of pKGW, 0 (Plant Systems Biology, Ghent, Belgium) for GOI expression, or 20 fmoles of supercoiled pDest/EFS destination vector for GOI silencing.

3. 1X Tris-EDTA (TE) Buffer, pH 8.0 (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), Proteinase K solution (2 μg/μL) provided by the LR clonase kit (Invitrogen, Carlsbad, CA, USA). Thermo-block or a water bath (Precisterm S-385) set at 37 ºC

2.5. *Agrobacterium tumefaciens* C58 transformation and agroinjection

1. Eppendorf tubes containing 40 μL of electro-competent *Agrobacterium* C58 cells stored at -80 ºC. Electroporator (Bio-Rad, gene-pulser 165-2077), 1 mm electroporation cuvettes (*Bio-Rad* Laboratories, CA, USA) and 15 mL tubes containing 250 μL of S.O.C. medium (2 % tryptone, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose). Sterile LB liquid medium and solid LB agar (LBA) plates containing 50 μg/mL Spectinomycin and 50 μg/mL Rifampicin antibiotics and a growing chamber set at 28 ºC. For *Agrobacterium* DNA minipreps use QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA).

2. *Agrobacterium* cultures and sub-cultures mediums for agroinjection: 15 mL tubes containing 3 mL of YEB medium (0.5 % beef extract, 0.1 % yeast extract, 0.5 % peptone, 0.5 % sucrose, pH 7.4), 2 mM MgSO4 and Spectinomycin and Rifampicin (50 μg/mL each) antibiotics. A shaker and a growing chamber set at 28 ºC.

3. Infiltration MES buffer: 10 mM MES (Sigma-Aldrich, MO, USA), 10 mM MgCl, 200 µM acetosyringone (Sigma-Aldrich, MO, USA). To prepare the 200 mM stock solution, dissolve 390 mg acetosyringone in 10 mL of dimethyl sulfoxide. Filter-sterilize and store at -20 °C. Spectrophotometer (UV/VIS Spectrophotometer SP8001, DINKO) set at a wavelength of 600 nm and transparent plastic cuvettes.

4. Tomato fruits (*Solanum lycopersicum cv.* Micro Tom) at a developing green stage 25 days post-anthesis (25 dpa) or in Mature Green (30-35 dpa), and sterile 1 mL Plastipak syringes with needle.

2.6. Fluorescence-based transient expression analysis.

1. Tomato fruits (*Solanum lycopersicum cv.* Micro Tom) agroinjected with *Agrobacterium* strains containing fluorescent protein constructs.

2. A Typhoon TRIO Variable Mode Imager (Amersham Biosciences, Piscataway, NJ, USA) fluorescence scanner (8610 or 9210 Typhoon model) with gel alignment guides and ImageQuant Tools software. A clean low-fluorescent glass plate, powder-free gloves and a meat cutter (CF-172 Fagor, Spain).

3. Methods

For our expression assays, we have used the pENFRUIT collection vectors which are based on the Three-Fragment Multisite Gateway PRO system and are publicly available in the Addgene repository (http://www.addgene.org/pgvec1) access numbers 20083-20107. The pENFRUIT collection is composed of three sub-collections of input vectors: (a) pEF1-PROM-4 containing promoter regions, (b) pEF4r-GOI-3r containing reporter genes, and (c) pEF3-TER-2 containing terminators and 3' marking sequences **(*8*)**. For

the triple recombination the pKGW,0 binary plasmid (Plant Systems Biology, Ghent, Belgium) is used as destination vector.

In the case of hpRNAi strategies, a fragment of the gene-of-interest (GOI) is cloned into pEFS4r-3r and pEFS3-2 vectors using two of the restriction enzymes that cut in the polylinker region (*Sac*I, *Sma*I and *Xho*I sites), which is set in reversed orientations in each plasmid. The GOI fragment is, in this way, inserted in inverted orientations in separate plasmids. A scheme of the strategies for a parallel ectopic expression and/or RNAi silencing of a GOI is shown in **Fig.1**.

3.1. BP cloning of a GOI for its expression in fruits: construction of a pEF4r-GOI-3r plasmid.

For the construction of a pEF4r-GOI-3r Entry vector, the GOI has to be amplified with oligonucleotide primers containing *att*B4r/*att*B3r recombination sites and incorporated into pDONR 221 P4r-P3r by BP mediated recombination.

1. Design forward and reverse oligonucleotides for your GOI template including 18-24 bp gene-specific sequences plus the sequences corresponding to *att*B4r (Fwd 5' GGGG ACA ACT TTT CTA TAC AAA GTT GNN) and *att*B3r (Rev 5'GGGG AC AAC TTT ATT ATA CAA AGT TGT) recombination sites (*see* **Note 1**).

2. Amplify your GOI sequence by PCR reaction using Advantage HD Polymerase Mix (Clontech, Mountain View, CA, USA) which is suitable for producing high fidelity and long amplicon size (until 8.5 kb).

3. Purify the amplified GOI by electrophoresis through a 1 % agarose gel. Prepare a thick TAE 1X minigel and charge the entire PCR reaction (50 µL) in a single well. Purify the DNA using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) obtaining 50 µL of elution volume. For large or difficult-to-clone GOI fragments, the use of Ethidium bromide and UV light should be avoided to reduce DNA

fragmentation. Quantify the DNA by using a Nanodrop spectrophotometer (Nanodrop ND-100 Spectrophotometer. Thermo Fisher Scientific Inc., USA). Alternatively, a polyethylene glycol (PEG) purification protocol is suggested by Invitrogen to purify *att*B PCR products (*see* **Note 2**).

4. Put 15-150 ng of your *att*B4r/*att*B3r purified GOI in a tube, add 1 µL of pDONR vector (pDONR 221 P4r-P3r, 150 ng) and TE Buffer to reach a total volume of 8 µL. BP Clonase II enzyme (Invitrogen, Carlsbad, CA, USA) is removed from -20 ºC, thawed on ice for 2 minutes and mixed briefly with the vortex. Add 2 µL of the enzyme to the BP reaction tube, mix by vortexing and incubate BP reaction tube at 25 ºC for 1-16 hours using a thermo-block (Thermolyne Dri-Bath Model 17600) (*see* **Note 3**). To stop the reaction 1 µL of Proteinase K solution is added and the tube is incubated at 37 ºC for 10 minutes.

5. Take a tube containing chemically competent *E. coli* cells, One Shot Mach1 T1 Competent Cells (Invitrogen, Carlsbad, CA, USA), from -80 ºC and thaw them on ice. Add 2 µL of BP reaction shaking the content gently and perform transformation following manufacturer's instructions. Collect 50-150 µL from the bacterial culture and spread in LB plates containing 50 µg/mL Kanamycin. Incubate the plates in a 37 ºC growing chamber for 16 hours.

6. Pick 5-10 colonies using sterilized toothpicks, grow them overnight in liquid media and obtain DNA minipreps from them using the E.Z.N.A.® Plasmid Mini Kit II (Omega Bio-tek, Doraville, GA, USA) Confirm the pEF4r-GOI-3r entry vector by restriction analysis or by sequencing using M13 forward and reverse primers.

3.2. "Classical" cloning of the GOI for silencing strategies: construction of pEFS4r-GOI-3r and pEFS3-IOG-2 vectors.

The pENFRUIT collection was adapted to hpRNAi strategies with the addition of two plasmids pENTR_RNAi (pEFS4r-3r and pEFS3-2) and the construction of a new Destination vector (pDest/EFS). Both pEFS3-2 and pEFS4r-3r vectors share the same cloning region (polylinker). This region contains *Sac*I, *Sma*I and *Xho*I sites and is set in reversed orientations in each plasmid. For hpRNAi strategies, a fragment of the GOI needs to be cloned into both pEFS4r-3r and pEFS3-2 using two of the restriction enzymes of the polylinker. The GOI fragment is, in this way, inserted in inverted orientations in separate plasmids. Between the two cloning sites, spans a fragment of a tomato intron which is subsequently removed by splicing. The pDest/EFS Destination vector, also incorporated in the collection, is suitable for hpRNAi strategies, as it contains a 35S terminator sequence downstream of the Gateway *att*R1-*att*R2 recombination sites. This enables the making of different constructions for silencing strategies driven by user-defined pEF1-4 promoters **(*8*)**. The types of vectors used for the expression or silencing of a GOI are summarized in **Table 1**.

1. Amplify a fragment of the GOI of approximately 100 bp in length usingoligonucleotides containing two different restriction sites from the polylinker (*SacI, SmaI or XhoI*) (*see* **Note 4**). PCR conditions will depend on fragment length and oligonucleotide Tm constrictions. The pEFS4r-3r, pEFS3-2 vectors and the GOI fragment are to be digested using the same enzymes in three different Eppendorf tubes in order to get "sticky ends". The GOI fragment can also be obtained from a vector by double enzymatic digestion using a combination of SacI, SmaI or XhoI restriction enzymes, thus avoiding the oligonucleotide design and amplification. Proper enzymatic

reaction conditions can be found at the enzyme manufacturer website (*see* **Notes 5 and 6**). An example of GOI fragment isolation is shown in **Fig. 2**.

2. Purify GOI fragment and pEFS4r-3r and pEFS3-2 linearized vectors using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA).

3. Prepare two 1.5 mL Eppendorf tubes and set two ligation reactions (pEFS4r-3r-GOI and pEFS3-2-GOI) using T4 DNA ligase (Invitrogen, Carlsbad, CA, USA) and the conditions for cohesive ends recommended by the manufacturer (Insert:Vector Molar Ratio of 3:1 and 23-26ºC). For instance, 57 ng (30 fmoles) of each pEFS vector (20 ng/µL) and 6 ng (90 fmoles) of 100 bp GOI fragment (19 ng/µL). After 1 hour or overnight ligation reaction, dilute the ligation volume 1/5 in each tube and proceed to transform *E. coli* chemically competent cells (*see* **Note 7**).

4. Proceed as described in 3.1.6, picking colonies and carrying out DNA minipreps. Confirm positive clones by *Sac*I, *Sma*I and *Xho*I restriction analysis. An example of gel confirmation is shown in **Fig.2**. Sequencing with M13 oligonucleotides may be required in order to confirm if the GOI has been cloned into pEFS4r-3r and pEFS3-2 vectors.

3.3. Three-Fragment Multisite Gateway LR recombination reactions: generation of pEXP_PR:GOI:T or pEXPhp P:GOI binary plasmids ready for *Agrobacterium* transformation

1. Prepare vectors required for the triple LR recombination reaction. For instance, in case of *GFP* expression driven by NH promoter, pEF1-NH-4, pEF4r-GFP-3r and pEF3-Tnos-2 Entry vectors and pKGW,0 destination vector would be used for the construction of pEXP NH:GFP:Tnos Expression vector. In another example, the gene silencing of *Rosea1* driven by the CaMV35S promoter would require pEF1-35S-4, pEFS4r-Ros1-3r and pEFS3-1soR-2 Entry vectors and pDest/EFS destination vector in order to make the pEXPhp NH:Ros1 Expression vector.

2. For each LR reaction, a 1.5 mL sterile Eppendorf empty tube is required. In every tube 10 fmoles of each Entry vector, 1 µL of the corresponding Destination vector (20 fmoles) and TE Buffer are added to reach a total volume of 8 µL. Them add 2 µL of LR Clonase II to each LR reaction tube, mix by vortexing and incubate at 25 ºC for 16 hours using a thermo-block (Thermolyne Dri-Bath Model 17600). To stop the reaction 1 µL of Proteinase K solution is added and tubes are incubated at 37 ºC for 10 minutes.

3. Transform chemically competent *E. coli* cells, One Shot Mach1 T1 Competent Cells (Invitrogen, Carlsbad, CA, USA) using 2 µL of LR reaction following manufacturer´s instructions (*see* **Note 8**). Plate cells in 50 µg/mL Spectinomycin LB agar plates. Put the plates in a 37ºC growing chamber for 16 hours.

4. Proceed as described in 3.1.6, by picking colonies and carrying out DNA minipreps. Analyze the DNA by restriction using the proper enzymes. For instance a digestion with an enzyme *Sac*I, *Sma*I or *Xho*I is appropriate in the case of Expression vectors for RNAi as it allows the isolation of the fragment corresponding to the haipin intron flanked by both GOI sequences. As an example, the construction of pEXPhp 35S:Ros1 vector aimed at silencing the *Rosea1* gene in transgenic tomato and tobacco plants is described in **Fig.2**. Additionally, the sequencing of the Expression vector can contribute to check if constructions have been correctly assembled. Similarly to BP reaction (*see* **Note 2**) the length of the fragments to recombine has a negative impact on the performance of the LR reaction (*see* **Note 9**).

3.4. *Agrobacterium tumefaciens* **C58 transformation.**

1. Add 1 µL from a positive *E. coli* plasmid miniprep of each construction to a sterile micro-centrifuge tube containing 40 µL of electro-competent cells of C58 *Agrobacterium tumefaciens* and electroporate samples using an electroporator (Bio-Rad,

gene-pulser model 165-2077) (*see* **Note 10**). Add 250 µL of SOC medium maintained at room temperature and incubate in 15 mL plastic cap tube at 28 ºC for 2 hours in agitation. Then, spread 50-150 µL of the bacteria suspension on solid LB plates containing 50 µg/mL Spectinomycin and 50 µg/mL Rifampicin antibiotics. Incubate for at least 48 hours at 28 ºC.

2. Check if the transformation has worked for every construction by picking 5-10 colonies from each culture plate (in sterile conditions) and incubate them in liquid LB culture containing Spectinomycin and Rifampicin (50 µg/mL each) antibiotics at 28 °C for at least 48 hours. Again, carry out DNA minipreps and digestions with the appropriate restriction enzymes to confirm the constructs (*see* **Note 11**).

3.5. Agroinjection of tomato fruits for transient expression assay: Analysis of promoter activity as an example.

Transient expression of genes can be used prior to stable transformation of plants to produce a large amount of protein or verify expression constructs **(*14*)**. A frequently used technique is agro-infiltration on plant leaves **(*13*)**, and we have used this technique to evaluate the functionality of some constructs as in the case of *Rosea1* RNAi Expression vector (*see* section **3.6.**). As we are more interested in tomato fruit biotechnology, our laboratory has developed a method based on the leaf agro-infiltration that was adapted to transient expression of foreign genes directly into the tissues of the fruit. The method implies the injection of a suspension of *Agrobacterium* cells in the fruit by using a syringe needle. The injection is made through the stylar apex of the fruit, so that the infiltrated solution reaches the entire surface of fruit **(*11*)**. We performed transient expression assays using *GFP* as reporter. The aim was to compare the activity of newly isolated fruit-specific promoters (for instance PNH), against ripening-related fruit promoters (such as E8) and against a constitutive promoter

(CaMV35) in two different stages of fruit ripening: Mature Green and Breaker. These promoter sequences were cloned next to a GFP/GUS translational fusion and tested in transient expression assays **(*8*)**. For the quantification of GFP fluorescence levels, though, the introduction of an additional fluorescent gene as internal standard like DsRed was necessary. For this purpose, the tomato fruits were co-injected with another *Agrobacterium* strain containing a CaMV35S:DsRed construct and tomato slices were simultaneously double-scanned to determine signal ratios between green and red fluorescence channels.

1. For every construction pick an *Agrobacterium* colony into a 50 mL culture tube containing 3 mL of YEB medium, 2 mM MgSO4 and Spectinomycin and Rifampicin (50 μg/mL each) antibiotics. Cultures are grown at 28 °C with shaking (150-200 rpm) for 36-48 hours inside a growing chamber.

2. Prepare a sub-culture by transfer 50 μL of the previous culture into a 50 mL tube containing 3 mL of YEB medium, 2 mM MgSO4 and Spectinomycin and Rifampicin (50 μg/mL each) antibiotics. Grow at 28 °C with shaking (150-200 rpm) for 16-24 hours.

3. Collect the *Agrobacterium* culture by centrifugation at 3000 rpm for 15 minutes using a centrifuge (Sigma-302) and resuspend it with infiltration MES buffer. Check the optical density (OD) and dilute if necessary adding more infiltration buffer to reach 0.05 OD (*see* **Note 12**). The optical density is measured at 600 nm wavelength by means of a spectrophotometer (UV/VIS Spectrophotometer SP8001, Dink). Put cultures to incubate at room temperature in gentle agitation (20 rpm) for at least 2 hours. GFP/GUS-containing cultures are collected and combined 1:9 (vol:vol) with an *Agrobacterium* C58 strain containing CaMV35S:DsRed construct (culture OD_{600}=0.05). Expression

assays can be improved by adding an *Agrobacterium* strain expressing the p19 silencing suppressor (*see* **Note 13**).

4. For the infiltration process, a 1 mL syringe with needle (25 GA 5/8 IN, needle: 0.5x16 mm, BD Plastipak™) is required for each mixed solution (construction/35S:DsRed). Infiltration is performed by inserting the needle about 3-4 mm within the tissue of tomato fruit (*Solanum lycopersicum cv.* Micro Tom) stylar apex and by injecting the solution carefully. The infiltrated solution can be visible as it spreads throughout the inner fruit tissues causing a darkening of the fruit. The agroinjection should finish when the fruit is fully infiltrated, at this stage a leakage of few drops is usually observed at the tip of the sepals. The amount of injected solution varies depending on the fruit size but 700 µL should be enough to infiltrate a whole full-grown Microtom fruit. Expression or silencing of de GOI can be observed between four and six days after infiltration. Usually the fruits are agroinjected after 25 days of the flower anthesis (25 days post-anthesis or 25 dpa) in the green phase. For studies of gene expression during fruit ripening, the fruit is agroinjected at a late Mature Green stage (30-35 dpa) and harvested at the Breaker/Red stage.

5. To determine the GFP/DsRed signal ratio a Typhoon TRIO Variable Mode Imager (Amersham Biosciences, Piscataway, NJ, USA) fluorescence scanner for large gels is required. Samples are to be placed onto a low-fluorescent glass plate which has to be previously cleaned with a 5 % DECON90 solution and 70 % isopropanol, rinsed with distilled water and dried thoroughly using a lint-free cloth (*see* **Note 14**).

6. Harvest six tomatoes for each construction between four and six days after infiltration and cut them using a meat cutter (CF-172 Fagor, Spain) to get 1 mm thick cross sections. Place the tomato slices (one or two for tomato) in a column distribution on the glass plate, so that each column corresponds to each construction and leave two

columns for 35S:DsRed and p19 control tomato cutlets. Put the glass into the Typhoon scanner and set the area and orientation using the Typhoon Scanner Control software. Use an excitation wavelength (λ_{ex}) of 532 nm and 610 nm emission filters (λ_{em}) to detect DsRed, and λ_{ex}=488 nm and a λ_{em}=520 nm filter to detect GFP simultaneously on a second channel. Pre-scan the glass plate first at low resolution (pixel size=500 µm) to make sure the experiment was successful and check the PMT voltage parameters to make sure the data is not saturated. Then scan the glass plate at higher resolution (pixel size=50 µm or 25 µm) and save the images.

7. Run the ImageQuant Tools software (Amersham Biosciences, Piscataway, NJ, USA) to open and analyze the images. For each tomato slice, modify a grid to select four circular areas with radii of 10 pixels each, located in the area of the placenta and/or gel showing DsRed fluorescence (*see* **Note 15**). Determine the ratios of fluorescence intensity of green (GFP) and red (DsRed) channels and put them in a table. Using an Excel spreadsheet represent the ratio values by subtracting the background noise, calculated as the average of the green/red ratios in those cutlets from tomatoes solely agroinjected with 35S:DsRed. Additionaly, to obtain more information on the expression patterns, constructs can be transferred to *Agrobacterium tumefaciens* LBA4404 strain by electroporation and used for tomato stable transformation **(*15*)**. An example of double-scanned tomato slices and Green/Red ratios representation of CaMV35S, PNH and E8 promoter activities is shown in **Fig. 3.** The GFP expression driven by 35S promoter in the green phase was the highest in the study of promoter activity in tomato fruit. In the Breaker phase, its activity decreased and was comparable to that of fruit-specific promoters. PNH promoter activity decreased during ripening, contrary to what was observed with the E8 promoter whose activity increased during the fruit ripening as described in the literature **(*16*)**.

3.6. Checking functionality of hpRNAi constructs: Transient silencing of *Rosea1* transgene as an example.

1. For transient hpRNAi experiments, transgenic Ros1 tobacco leaves constitutively expressing *Anthirrynum majus Rosea1* gene were syringe-infiltrated with an *Agrobacterium* culture (OD=0.05) carrying pEXhp 35S:Ros1, following a standard leaf agroinfiltration protocol as described in **(*13*)**. Ros1 plants show a bronze-colored phenotype as results of the activation of flavonoid biosynthesis promoted by Rosea1 transcription factor (unpublished). The successful silencing of *Rosea1* transgene in a hpRNAi strategy is easily detectable as a reversion to the green wild-type phenotype. Three week later the phenotype of the infiltrated leaves was scored as represented in **Fig. 4A**.

2. A similar strategy was followed using an anthocyanin-rich transgenic tomato line derived from F6DR line **(*17*)** carrying *Rosea1* and *Delila* transgenes **(*18*)** An *Agrobacterium* culture (OD=0.05) carrying pEXhp 35S:Ros1 was agroinjected in mature green fruits as described in the previous section. Here, agroinjection of pEXhp 35S:Ros1 construct should result in a partial reversion from light purple to the red (wild type) tomato color. The result of the silencing experiments was recorded three weeks after agroinjection and is shown in **Fig. 4B**.

4. Notes

1. Because of their length, we recommend ordering highly purified *att*B4r/*att*B3r oligonucleotides (HPLC or PAGE purification). We got recombination problems or obtained no PCR product at all when using desalted only oligonucleotides.

2. We have confirmed that high frequency of A/T nucleotides (in promoter sequences for instance) affects BP reaction yield. We have followed PEG-based purification protocol (as suggested by Invitrogen to remove *att*B primer-dimers), as a substitute of gel purification in some cases of unsuccessful BP reaction and we obtained good results. Usually a 30 % PEG/Mg solution is supplied in BP Clonase II kits.

3. Invitrogen suggests BP incubations longer than 1 hour for large PCR products (≥5 kb). We have confirmed an increase in the yield of colonies after overnight incubations, however a good yield can be obtained with incubations of 1 or 2 hours for 1-2 kb PCR products.

4. Post-transcriptional gene silencing (PTGS) is produced by small RNAs, and has been proved that fragments of the gene of interest of approximately 100 bp are enough to trigger an efficient PTGS response **(*19, 20*)**.

5. An hour of enzymatic digestion may be enough, but these restriction enzymes are suitable for an overnight digestion in case of poor band yielding.

6. To ensure the linearization of pEFS vectors we suggest an overnight enzymatic digestion. A treatment of 30 minutes (37 ºC) of vector molecules with calf intestinal alkaline phosphatase (Promega Corporation, Madison, USA) to remove the 5'-phosphate groups can be used to prevent re-circularization.

7. Although the manufacturer suggests that 1 hour of ligation reaction is sufficient in case of cohesive ends, we have obtained better results with an overnight reaction at 24 ºC. We have also found that a 1/5 dilution of the ligation reaction is more efficient in *E. coli* transformation as suggested by the manufacturer.

8. You can use one type or another of *E. coli* competent cells, both showed similar transformation efficiencies. However, the T1 Match cells are recommended by Invitrogen for their faster growing rate.

9. We have checked that overnight LR reactions increase the number of colonies containing the desired expression clone. Also for large (>10 kb) Entry clones or Destination vectors, linearizing the Entry clone or Destination vector increase the LR efficiency as pointed by Invitrogen.

10. In tomato, *Agrobacterium* C58 strain is more efficient in transient expression assays whilst other strains like LBA 4404 for instance, are more suitable for plant stable transformation.

11. To improve *Agrobacterium* plasmid minipreps yield of low copy number plasmids, pellet the cells from 10 mL liquid LB medium and follow the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA, USA) procedure. Plasmid yield improves by adding 20 µL of phosphatase alkaline (CIP) (New England Biolabs, Hitchin, UK) to the column and incubating the column in ice for 15 minutes prior to the miniprep's washing step.

12. Experimental assays performed at different ODs have proved that OD_{600} values of 1.0 to 0.5, resulted in low reporter gene (for instance *GFP*) expression levels. We have concluded that a more diluted bacteria culture (0.05 to 0.005 OD_{600} values) results in a better distribution of GFP fluorescence in the fruit.

13. PTGS can be reduced by co-expression with a suppressor of viral proteins, such as p19 protein **(*21*)**. In our lab, tomato fruits have shown higher expression values and longer time stability of the protein of interest, when GFP/GUS expressing cultures were

co-injected 1:20 (vol:vol) with pBIN 61 P19 (OD_{600}=0.05) *Agrobacterium* p19-expressing strain.

14. Dust fluoresces and scatters light, which causes artefacts on images and can interfere with quantification. To avoid this, wear powder-free gloves and use clean and rinsed low-fluorescent glass plates.

15. The highest accumulation of GFP protein takes place in the placental tissue which is more accessible to the bacteria. In some cases, fluorescence can be observed in the inner surface of the pericarp, however this tissue remains impregnable and should be studied by other techniques.

5. Acknowledgements

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6. Figure Captions

Fig.1. Scheme representing the necessary steps for the expression and the silencing of a GOI. GOI expression requires, as a previous step, the making of pEF4r-GOI-3r Entry vector by BP recombination, whilst the GOI silencing requires the classic cloning of a GOI fragment in the pEFS4r-3r and pEFS3-2 vectors. These steps are prior to LR

recombination reaction which in case of the GOI expression requires Entry vectors containing the promoter and terminator sequences (pEF1-PROM-4 and pEF3-TER-2) and the pKGW.0 Destination vector. For the GOI silencing, pEFS1-PROM-4 Entry vector adding the promoter sequence, and the pDest/EFS Destination vector are needed. The viability of the resulting Expression vectors is checked by transient expression in tomato fruits (*see* section **3.5.**) using the agroinjection technique.

Fig.2. Isolation of *Rosea1* 100 bp gene fragment (Ros1) and cloning into pEFS4r-3r and pEFS3-2 vectors. (**A**) Ros1 fragment was isolated after 3 hours of double enzymatic digestion with XhoI/SacI at 37 ºC from 6.2 µg pCR8ROS plasmid DNA. The same double digestion conditions were used to get pEFS4r-3r and pEFS3-2 linearized plasmids, in this case from 2.04 µg and 3.38 µg respectively. (**B**) Ros1 100 bp fragment and the linearized pEFS vectors were gel extracted and purified. Two parallel T4 Ligation reactions were performed overnight at 24 ºC which required 5.94 ng (90 fmols) of purified Ros1 fragment DNA, and 58 ng (30 fmols) of both linearized pEFS vectors. After *E. coli* transformation, eight colonies were grown (4 for each ligation) and 20 µL of each plasmid miniprep was double digested with XhoI/SacI at 37 ºC for 1 hour. Enzymatic digestions were checked on a 1.5 % agarose TAE 1X electrophoresis gel which revealed the 100 bp Ros1 fragment in seven of eight digestions. (**C**) After a LR recombination reaction using pEF1-CaMV35S-4, pEFS4r-Ros1-3r, pEFS3-1soR-2 and pEFS/Dest vectors, the pEXPhp 35S:Ros1 Expression vector was obtained. As a result of *SacI* digestion two fragments were produced, one of 9757 bp and another of 709 bp, this latter corresponding to the tomato intron flanked by 100 bp *Rosea1* gene sequences. *SacI* did not cut pEFS/Dest and linearized pEFS4r-3r and pEFS 3-2 vectors.

Fig.3. Promoter analysis using transient expression. (**A**) Examples of scanning images obtained from sliced tomatoes transiently transformed with promoter:reporter

constructs. Control tomatoes were agroinjected with a non-fluorescent construct. 35S:DsRed is used as an internal standard. (**B**) Green/red fluorescence ratios from promoters CaMV35S, PNH and E8 measured at mature green (empty columns) or breaker (filled columns) stages. For each tomato slice, four circular areas corresponding to placenta and/or gel areas showing DsRed fluorescence were selected. Background values (from CaMV35S:DsRed injected only) were subtracted from the final values. Bars represent average ratios ± standard deviation.

Fig.4. *Rosea1* gene transient hpRNAi silencing in 35S:*Rosea1* (Ros1) tobacco leaves (**A**) and E8:Rosea1-E8:Delila tomato fruits (**B**). **IA**: Infiltrated areas of the organ; **NA**: Non-Infiltrated Areas of the same organ (only applicable to leaves, where infiltration zone can be visually monitored at the time of infiltration); **C**: Control, non-infiltrated organ.

7. Table Caption

Table 1. Types of Gateway vectors used in the three fragment Multisite recombination reaction for the construction of Expression vectors aimed to express or silence a gene of interest in plants. **PROM**: Promoter sequence, **GOI**: Gene of interest and **TER**: Terminator sequence.

8. Tables

Vector	Type	Recombination sites	Application	Goal
pDONR 221 P4r-P3r	Donor vector	*att*P1/*att*P4	BP cloning	GOI expression
pEF1-PROM-4	Entry vector	*att*L1/*att*L4	LR cloning	GOI expression
pEF4r-GOI-3r	Entry vector	*att*L4r/*att*L3r	LR cloning	GOI expression
pEF3-TER-2	Entry vector	*att*L3/*att*L2	LR cloning	GOI expression
pKGW,0	Destination vector	*att*R1/*att*R2	LR cloning	GOI expression
pEFS4r-GOI-3r	Entry vector	*att*L4r/*att*L3r	LR cloning	GOI silencing
pEFS3-IOG-2	Entry vector	*att*L3/*att*L2	LR cloning	GOI silencing
pDest/EFS	Destination vector	*att*R1/*att*R2	LR cloning	GOI silencing
pEXP PROM:GOI:TER	Expression vector	*att*B1/*att*B2	Expression in Plant	GOI expression
pEXPhp PROM:GOI	Expression vector	*att*B1/*att*B2	Expression in Plant	GOI silencing

9. References

1. Davuluri, G. R., van Tuinen, A., Fraser, P. D., Manfredonia, A., Newman, R., Burgess, D., Brummell, D. A., King, S. R., Palys, J., Uhlig, J., Bramley, P. M., Pennings, H. M., and Bowler, C. (2005) Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes, *Nat Biotechnol* **23**, 890-895.
2. Davuluri, G. R., van Tuinen, A., Mustilli, A. C., Manfredonia, A., Newman, R., Burgess, D., Brummell, D. A., King, S. R., Palys, J., Uhlig, J., Pennings, H. M., and Bowler, C. (2004) Manipulation of DET1 expression in tomato results in photomorphogenic phenotypes caused by post-transcriptional gene silencing, *Plant J* **40**, 344-354.
3. Van Haaren, M. J., and Houck, C. M. (1993) A functional map of the fruit-specific promoter of the tomato 2A11 gene, *Plant Mol Biol* **21**, 625-640.
4. Hartley, J. L., Temple, G. F., and Brasch, M. A. (2000) DNA cloning using in vitro site-specific recombination, *Genome Res* **10**, 1788-1795.
5. Curtis, M. D., and Grossniklaus, U. (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta, *Plant Physiol 133*, 462-469.
6. Karimi, M., Bleys, A., Vanderhaeghen, R., and Hilson, P. (2007) Building blocks for plant gene assembly, *Plant Physiol* **145**, 1183-1191.
7. Karimi, M., Inze, D., and Depicker, A. (2002) GATEWAY vectors for Agrobacterium-mediated plant transformation, *Trends Plant Sci* **7**, 193-195.
8. Estornell, L. H., Orzaez, D., Lopez-Pena, L., Pineda, B., Anton, M. T., Moreno, V., and Granell, A. (2009) A multisite gateway-based toolkit for targeted gene expression and hairpin RNA silencing in tomato fruits, *Plant Biotechnol J* **7**, 298-309.
9. Fernandez, A. I., Viron, N., Alhagdow, M., Karimi, M., Jones, M., Amsellem, Z., Sicard, A., Czerednik, A., Angenent, G., Grierson, D., May, S., Seymour, G., Eshed, Y., Lemaire-Chamley, M., Rothan, C., and Hilson, P. (2009) Flexible tools for gene expression and silencing in tomato, *Plant Physiol* **151**, 1729-1740.
10. Sasaki, Y., Sone, T., Yoshida, S., Yahata, K., Hotta, J., Chesnut, J. D., Honda, T., and Imamoto, F. (2004) Evidence for high specificity and efficiency of multiple recombination signals in mixed DNA cloning by the Multisite Gateway system, *J Biotechnol* **107**, 233-243.

11. Orzaez, D., Mirabel, S., Wieland, W. H., and Granell, A. (2006) Agroinjection of tomato fruits. A tool for rapid functional analysis of transgenes directly in fruit, *Plant Physiol* **140**, 3-11.
12. Wroblewski, T., Tomczak, A., and Michelmore, R. (2005) Optimization of Agrobacterium-mediated transient assays of gene expression in lettuce, tomato and Arabidopsis, *Plant Biotechnol J* **3**, 259-273.
13. Kapila, J., De Rycke, R., Van Montagu, M., and Angenon, G. (1997) An Agrobacterium-mediated transient gene expression system for intact leaves, *Plant Science* **122**, 101-108.
14. Twyman, R. M., Stoger, E., Schillberg, S., Christou, P., and Fischer, R. (2003) Molecular farming in plants: host systems and expression technology, *Trends Biotechnol* **21**, 570-578.
15. Ellul, P., Garcia-Sogo, B., Pineda, B., Rios, G., Roig, L. A., and Moreno, V. (2003) The ploidy level of transgenic plants in Agrobacterium-mediated transformation of tomato cotyledons (Lycopersicon esculentum Mill.) is genotype and procedure dependent [corrected], *Theor Appl Genet* **106**, 231-238.
16. Deikman, J., Kline, R., and Fischer, R. L. (1992) Organization of Ripening and Ethylene Regulatory Regions in a Fruit-Specific Promoter from Tomato (Lycopersicon esculentum), *Plant Physiol* **100**, 2013-2017.
17. Orzaez, D., Medina, A., Torre, S., Fernandez-Moreno, J. P., Rambla, J. L., Fernandez-Del-Carmen, A., Butelli, E., Martin, C., and Granell, A. (2009) A visual reporter system for virus-induced gene silencing in tomato fruit based on anthocyanin accumulation, *Plant Physiol* **150**, 1122-1134.
18. Butelli, E., Titta, L., Giorgio, M., Mock, H. P., Matros, A., Peterek, S., Schijlen, E. G., Hall, R. D., Bovy, A. G., Luo, J., and Martin, C. (2008) Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors, *Nat Biotechnol* **26**, 1301-1308.
19. Ossowski, S., Schwab, R., and Weigel, D. (2008) Gene silencing in plants using artificial microRNAs and other small RNAs, *Plant J* **53**, 674-690.
20. Wesley, S. V., Helliwell, C. A., Smith, N. A., Wang, M. B., Rouse, D. T., Liu, Q., Gooding, P. S., Singh, S. P., Abbott, D., Stoutjesdijk, P. A., Robinson, S. P., Gleave, A. P., Green, A. G., and Waterhouse, P. M. (2001) Construct design for efficient, effective and high-throughput gene silencing in plants, *Plant J* **27**, 581-590.
21. Voinnet, O., Rivas, S., Mestre, P., and Baulcombe, D. (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus, *Plant J* **33**, 949-956.

Figure 1

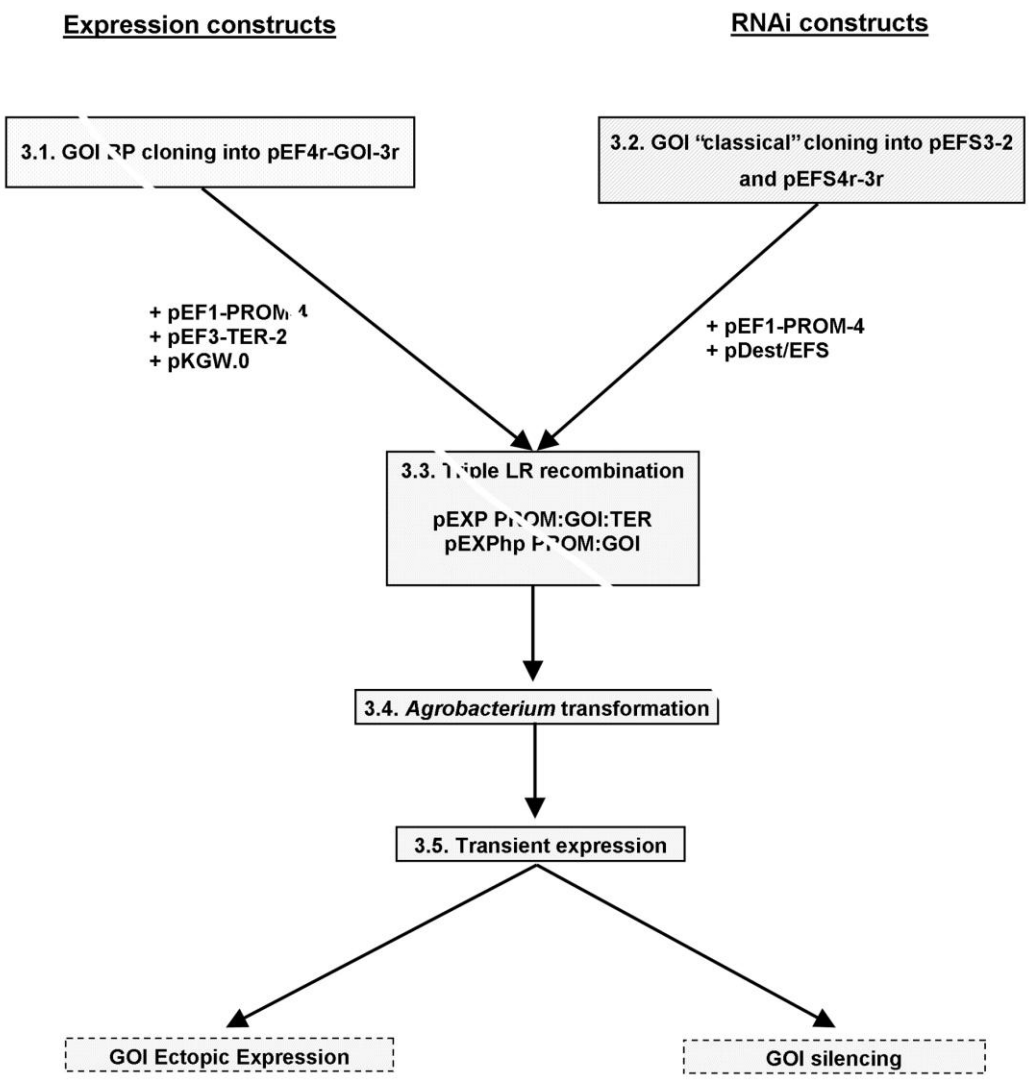

Expression constructs
RNAi constructs
3.1. GOI GP cloning into pEF4r-GOI-3r
3.2. GOI "classical" cloning into pEFS3-2 and pEFS4r-3r
+ pEF1-PROM-4
+ pEF3-TER-2
+ pKGW.0
+ pEF1-PROM-4
+ pDest/EFS
3.3. Triple LR recombination
pEXP PROM:GOI:TER
pEXPhp PROM:GOI
3.4. Agrobacterium transformation
3.5. Transient expression
GOI Ectopic Expression
GOI silencing

Figure 2

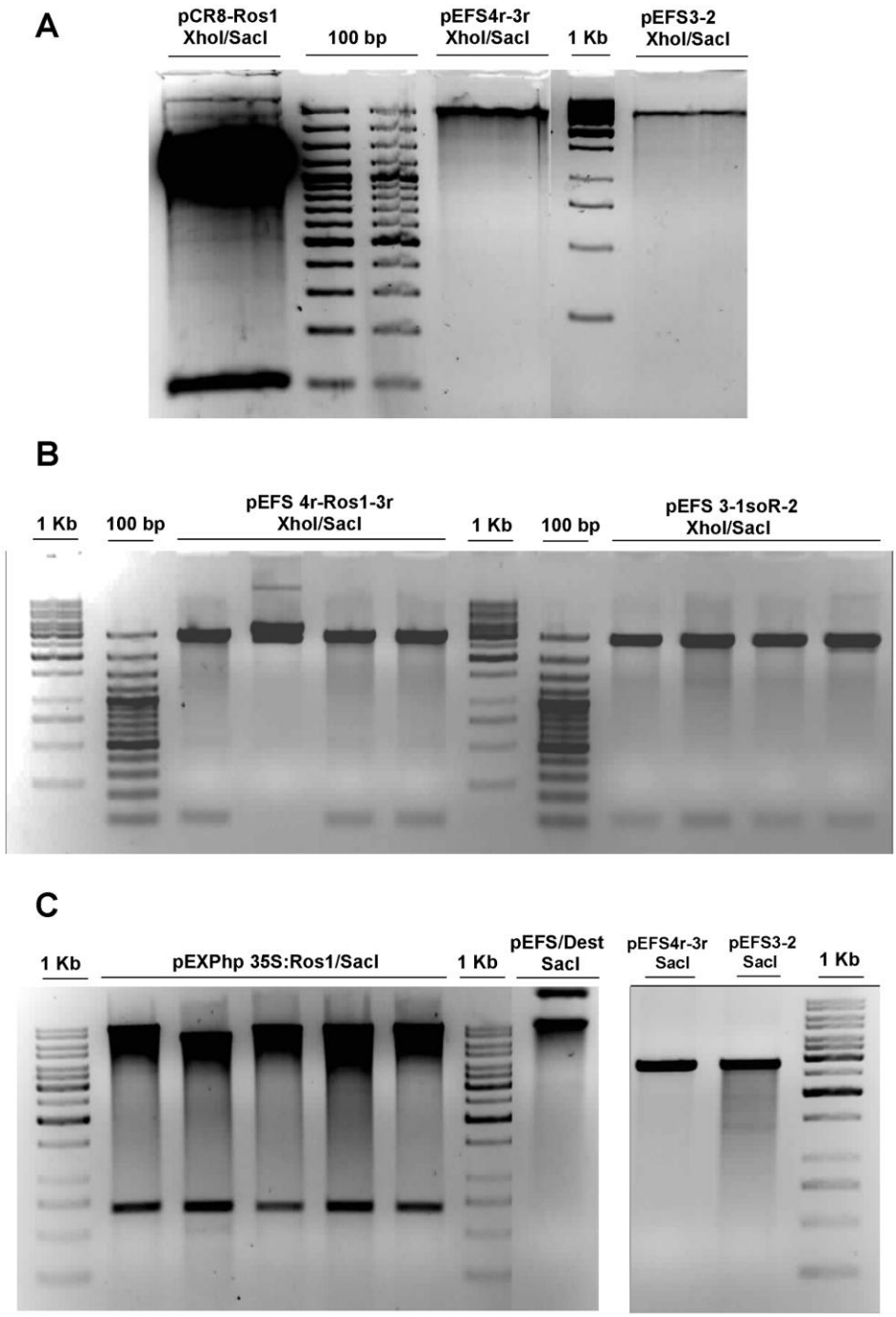
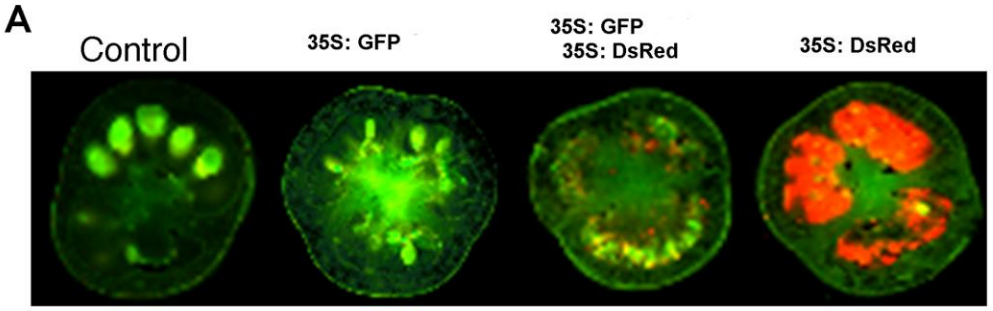

A
pCR8-Ros1 XhoI/SacI
100 bp
pEFS4r-3r XhoI/SacI
1 Kb
pEFS3-2 XhoI/SacI
B
1 Kb
100 bp
pEFS 4r-Ros1-3r XhoI/SacI
1 Kb
100 bp
pEFS 3-1soR-2 XhoI/SacI
C
1 Kb
pEXPhp 35S:Ros1/SacI
1 Kb
pEFS/Dest SacI
pEFS4r-3r SacI
pEFS3-2 SacI
1 Kb

Figure 3


A
Control
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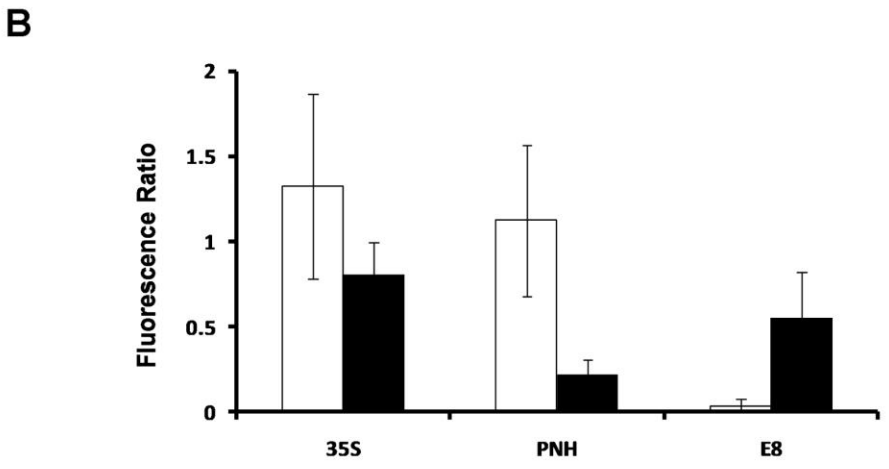
35S: GFP
35S: DsRed
35S: DsRed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Fluorescence Ratio
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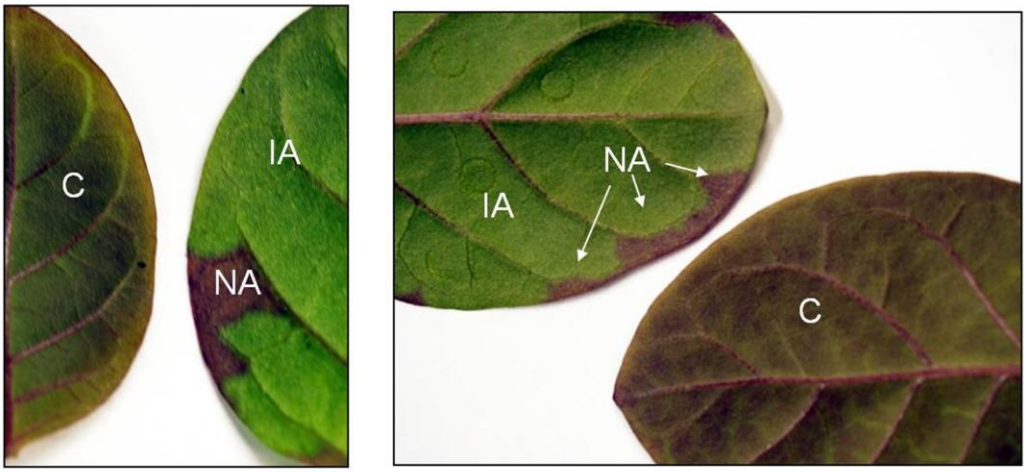
1
0.5
0
35S
PNH
E8

Figure 4

A



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